Contribution to the biology of the organism causing leguminous tubercles.

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WITH PLATES XII–XV.

(Concluded from p. 237.)

Comparative review.

This brings the history of the more important contributions to the biology of the leguminous tubercle organism, that have come to the notice of the present writer, down to the present time. The record presents a discouraging volume of conflicting testimony, some of it from eminent investigators. It would indeed be a misfortune should all these painstaking and laborious investigations really be so much at variance as appears from this examination of the contributions. Some of them which deny any external agent of a microbic nature will always remain important expositions of the structure and development of the tubercles. Had more attention been given by these investigators to careful cultural experiments perhaps they might have come to different conclusions. The charge might perhaps be made that cultural experiments are untrustworthy since Tschirch[97] and Frank[98] assert that sterilizing the soil by heat so changes its physical condition as to interfere with the development of the tubercles. Frank[99] and Schindler[100] also believe that the formation of the tubercles stood in direct relation to the vigor or assimilatory activity of the plants. Since plants do not usually grow so well under cultural conditions this might be said to argue against the trustworthiness of cultural experiments. In a large series of cultural experiments designed especially to test the correctness of these suggestions Prazmowski found them to be groundless. The cultural experiments carried on by the present writer show that sterilizing the soil, or a low state of vigor in the plants, will not prevent the development of the tubercles if the proper organism is given access to the roots.

In view of all the cultural experiments referred to above,

[97] Bot. Centralb. XXXI (1887).

[98] Ueber den Einfluss, welchen das Sterilisieren des Erdbodens auf die Pflanzenentwickelung ausübt. Ber. d. deutsch. bot. Gesells. VI (1888). *XCV.*

[99] Untersuchungen über die Ernährung der Pflanze mit Stickstoff. Landw. Jahrb. XVIII (1888). 496.

[100] Jour. für Landwirtschaft. XXXIII (1885). 330.

and in addition those carried on by Atwater,[101] Bréal,[102] Bertholet,[103] and Vines,[104] conviction becomes inevitable that leguminous plants can only develop tubercles when excited by the presence of certain micro-organisms.

The important question then is, can these various conflicting notions of the biology of the microsymbiont be harmonized? Leaving out of consideration for the present the real nature of the organism it will be admitted by those who take the trouble to familiarize themselves with the scope of the work covered by the most important investigations that the organism in question consists of an elongated thread-like structure, which branches freely within the tubercle and possesses enlarged portions which present a more or less finely lobed surface; and very much smaller forms which must exist to some extent within the tubercle, are capable of multiplying in artificial media, and, when transplanted from artificial media to the roots of leguminous plants, are capable, under these more natural conditions and the stimulus of the macrosymbiont, of growing out again into the thread-like structures.

Beyerinck[105] then probably overlooked the real nature of the thread-like structures. From a careful study of his illustrations and descriptions it seems reasonably certain that, in some instances at least, he was dealing with the true organism in his artificial cultures. An examination of his figure II *C* shows the organism to be very similar in form to those in my own cultures represented in figures 11 and 12, plate XIV, and to those obtained by Laurent. In describing them he says: "Die Colonien auf Gelatine bestehen aus stark gebuckelten bacteroidenähnlichen Stäbchen." The culture of this one was obtained from tubercles of *Vicia hirsuta.*

Recently Nobbe, Schmid, Hiltner and Hotter,[106] while considering the organisms to be bacteria, admit that in the

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[101] Atmospheric nitrogen as plant food. Bull. no. 5, Storrs' School Agr. Exp. Station, Conn. Oct. 1889.

[102] Fixation de l'azote par les légumineuses. Compt. Rend. herbd. d. Sci. d. l'Acad. d. Sciences, Paris. CIX. Oct. 28. 1889.

[103] Experiénces nouvelles sur la fixation de l'azote par certain terres végétales et par certaines plantes. Ann. d. Chim. et d. Phys. VI. XVI. Avril, 1889.

[104] On the relation between the formation of tubercles on the roots of Leguminoseæ and the presence of nitrogen in the soil. Ann. Botany II (1888–1889). 386–388.

[105] Bot. Zeit. 1888.

[106] Versuche über die Stickstoff-Assimilation der Leguminosen. Landw Versuchs-Stationen. XXXIX (1891).

cultures, especially from tubercles of *Lupinus*, numerous larger bacteroid forms developed. "Wir haben jedoch in unseren mehrfachen Uebertragungen Gebilde oft in grosser Anzahl gefunden, welche unzweifelhaft als echte Bakteroiden angesprochen werden mussten."

The tests imposed by Eriksson,[107] Ward,[108] Vuillemin,[109] Pichi,[110] A. Koch,[111] and Laurent[112] for the determination of the presence of a membrane in connection with the thread-like structures, would seem to impeach Prazmowski's[113] and Frank's[114] explanations of its nature. H. Möller[115] also finds a membrane on the strands but interprets it as being a cellulose membrane deposited by the protoplasm of the leguminous plant around the bacterian zoogloea, and cites in support of this view cellulose membranes said by R. Wolff[116] to be deposited about the threads of *Ustilagineæ* by their hosts. Such a view does not seem to be any greater proof of the bacterian nature of the organism of the tubercles than of a like nature for the *Ustilagineæ*.

It is difficult also to harmonize Prazmowski's description of the organism in artificial cultures with that obtained by the majority of those who have succeeded in growing it outside of the tubercles. It would, perhaps, be unjust to infer that his cultures were contaminated; or shall we suspect him of committing the same error which he imputed to Beyerinck, *viz.*, that he was deceived in the appearance of the forked or lobed condition of the organism? Prazmowski says this appearance might be produced by one rod lying partly over another, since frequently he was for a time deceived by such appearances. Could we not then, on the same ground say that the bacteroids are not forked?

It was impossible in the case of the cultures obtained by the present writer to be deceived in the form of the organism. The rod-like forms were exceedingly rare. It would seem that Laurent's work was carefully guarded for Metchnikoff

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[107] Bot. Zeit. 1874.
[108] Phil. Trans. Royal Soc. CLXXVIII; Proceed. Roy. Soc. XLVI.
[109] Ann. d. Sci. Agr. Franç. et etrang. 1888.
[110] Atti d. Soc. Toscani d. Sci. nat. 1888.
[111] Zur Kenntniss der Fäden in den Wurzelknöllchen. Bot. Zeit. 1890.
[112] Ann. d. l'Inst. Pasteur. V (1891).
[113] Landw. Versuchs-Stationen XXXVII (1890).
[114] Ueber die Pilzsymbiose der Leguminosen. Berlin, 1890.
[115] Ber. d. deutsch. bot. Gesells. X (1892). 242–249.
[116] Brand d. Getreides, 1874.

himself observed some of the lobed organisms in his cultures.

There does not seem to be any very important difference between the organisms described by Laurent and that obtained by myself. The result is the more satisfactory since the present writer did not know, at the time the organism was separated and first studied, what the real nature of Laurent's organism was. The account of the organism given by Ward agrees in all essential features with the one obtained by Laurent and myself.

Frank's mycoplasm of the tubercles is identical with the hyphæ, of course, but the reconciliation of his *Rhizobium*, a micrococcoid organism, with the forms obtained in culture by Beyerinck, Laurent and myself is not so easily effected, though the great majority of the individuals in the cultures from the tubercles of *Vicia sativa* were very small and without very high magnifying power would appear micrococcoid. The study of the form of the organism in the cultures was made with the aid of a Winkel microscope, the $\frac{1}{24}$ homogeneous immersion lens being used. A Zeiss 2^{mm} homogeneous immersion lens also served very well to bring out the definition of the form. Better results were obtained in examining the organism in a living condition, or by staining in a living condition with eosin. Killing and fixing the organism by heat on the cover glass did not give such good results because of the lack of firmness in the body of the organism.

Another question which arises, and which, if answered in the affirmative, may help to explain some of the discrepancies between the organisms in cultures by different investigators, is this: are there species or races of the microsymbiont? The bacteroids, by those who believe in the presence of a microsymbiont, are generally accepted as one form of the organism. They are regarded by Prazmowski as involution forms, because of their departure from the normal forms of rod-like bacteria. Whether or not we term them forms of involution it seems pretty certain that, when the organism has reached the firmness exhibited by the great mass of bacteroids in the tubercles, they are no longer capable of growth, since they have lost that power in becoming receptacles for the storage of proteid substance.

Prazmowski says the death of the bacteria is first announced in most cases by a change to the branched form. It would

probably be more nearly correct to say that the death of the organism, in its passage to the sterile condition of the perfect bacteroids is first indicated by a firmer condition of the organism, probably brought about by the increasing presence of proteid matter which in many cases finally becomes centered in different parts of the bacteroids and forms bodies which possess a very high power of refracting light. Lundström[117] described these in the tubercles of *Trifolium repens.* In some cases these bodies occupy nearly the entire inner portion of the bacteroids and frequently the accumulation takes place to such an extent as to cause the form of the bacteroid to enlarge, when, if there are several such bodies in an elongated bacteroid it presents a nodulose appearance as shown in some of the bacteroids from *Medicago denticulata* in figures 14 and 15 of plate XIV. In the bacteroids of species of Trifolium frequently the great increase in the size of these bodies gives to them the form of a bladder, and Beyerinck[118] has designated them as "Bläschenbacteroiden." Prazmowski shows that these bodies in the bacteroids do not take such stains as methyl violet. The present writer has observed that they do not take the stains gentian violet and fuchsin. On staining bacteroids from tubercles of *Medicago denticulata* with fuchsin they present an interrupted stain, simulating in this respect the rods of *Bacillus tuberculosus.* It is quite likely that the difficulty experienced in staining these objects in the tubercles has led some to describe the stained portions as spores.

Prazmowski calls attention to the fact that concentrated sulphuric acid will not dissolve normal bacteria, but that it will dissolve these highly refringent bodies in the tubercles giving to them a rose red color, which he claims shows them to be proteid bodies. Recently Frank[119] places them with the starch group calling them amylodextrin bodies while H. Möller,[120] in reply says they represent some form of cholesterin. In this same paper Frank states that he has discovered a dimorphism in the tubercles on the roots of peas, that the large profusely forked ones bear principally these

[117] Bot. Centralb., XXXIII (1888).

[118] Bot. Zeit. 1888.

[119] Ueber den Dimorphismus der Wurzelknöllchen der Erbse. Berichte d. deutsch. bot. Gesellsch. x (1892). 170–178.

[120] Bemerkungen zu Frank's Mittheilung über den Dimorphismus der Wurzelknöllchen der Erbse. Ber. d. deut. bot. Ges. x (1892). 242, 249.

amylodextrin bacteroids, while the smaller simple forms bear principally proteid bacteroids. H. Möller takes exception to this statement also, while Frank[121] refutes Möller's objection on the ground that Möller's study was confined to the tubercles of *Trifolium*, while Frank's announcement of a dimorphism related to the tubercles on *Pisum*.

There are many different forms of bacteroids associated with the tubercles of different species or genera of leguminous plants. As noted above Schneider has based several species of his *Rhizobium* purely on these characters of form and the more or less definite localization of the protoplasm at various points of the accumulation of these highly refringent and not readily stained bodies.

Morck,[122] while not describing them as species, figured numerous forms from tubercles of between forty and fifty species of *Leguminosæ*.

While these bacteroids are incapable of growth they may represent to a certain extent morphological characters of the organism within the tubercles. If this be true it would strengthen the proposition suggested by different forms obtained in artificial cultures that there are different varieties, or races, of the organism.

Schrœter[123] describes two species of his *Phytomyxa* based on the presence or absence of the strands in the tubercles. Some investigations of Hellriegel, Laws and Gilbert, Prazmowski (l. c.), point to a probability that lupines will not develop tubercles when seeded with soil-extract from places where lupines have not grown, while peas, etc., seeded with the same soil-extract develop the tubercles.

Beyerinck (l. c.) claims that in his artificial cultures different races were obtained which remained true to form through successive cultures.

Nobbe, Schmid, Hiltner and Hotter[124] found that *Lupinus luteus* inoculated with pea tubercle organisms, as well as those from *Robinia*, *Cytisus* and *Gleditschia*, developed no tubercles, but when inoculated with lupine tubercle organisms, devel-

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[121] Ueber Möller's Bemerkungen bezüglich der dimorphen Wurzelknollchen der Erbse. Ber. deutsch. bot. Gesells. x (1892). 390–395.

[122] Ueber die Formen der Bakteroiden bei den einzelnen Spezies der Leguminosen. Inaug-Dissert. Leipzig, 1891.

[123] Kryptogamen-Flora von Schlesien, 134.

[124] Versuche über die Stickstoff-Assimilation der Leguminosen. Landw. Versuchs-Stationen xxxix (1891). 227–359.

oped tubercles. *Phaseolus vulgaris* inoculated with cultures from tubercles of *Phaseolus* and peas developed tubercles, but if inoculated with cultures from tubercles of *Lupinus* or *Robinia*, none were developed. In one case *Pisum sativum* inoculated with lupine tubercle organisms developed tubercles, while in other cases it did not. In the case where the tubercles were developed, the hyphæ and bacteroid characteristic of those of the peas under normal conditions were developed. If this development of tubercles on peas from lupine organisms were not an accidental contamination it would indicate that one and the same species occurred in the tubercles of peas and lupine. Other cross inoculations made by them occasionally took effect but there was shown a disposition to tardy and weak development as if the organism had been in some unsuitable condition.

At the same time that the present writer carried on the second experiment in the inoculations of *Vicia sativa* with artificial cultures of the vetch tubercle organism, inoculations were also made of young plants of *Dolichos sinensis* with organisms from the same culture but no tubercles were developed while the inoculated plants of *Vicia sativa* developed tubercles.

But considering the almost universal infection of leguminous plants when grown in a state of nature it is difficult to believe that there are so many species as are represented by the different forms of bacteroids. Rather at the present time the question might be asked, does not the influence of the macrosymbiont upon the microsymbiont while within the tubercle fix a certain type of racial form and attenuation upon the microsymbiont until it shall have passed through normal conditions in the soil again and been restored to its original form and infecting power? The system of preventive inoculation depends largely upon the development of racial peculiarities and degrees of attenuation obtained by growing organisms in the presence of some deleterious substance, by cultivating them at certain temperatures for given periods of time, subjecting them to various degrees of atmospheric pressure, as by passing them through the bodies of other animals than those in which the organism is at first so virulent. While it is maintained by some that these racial peculiarities artificially produced remain fixed, there is evidence to show that after a time the attenuated organism may, by being subject

to certain normal influences, gradually regain its pristine characteristics and virulence.

This suggestion is only offered as a possible hypothesis for the explanation of apparent racial peculiarities in the microsymbionts of the tubercles. It must stand or fall only by a very comprehensive and thoroughgoing investigation. If it should be proven to be a correct one it would help to explain some of the conflicting observations upon the morphology of the organism.

Its solution one way or the other must be the crowning result of the remarkable series of investigations that have thus far contributed to a knowledge of one of the most abstruse problems of biological science, and would give a firm foundation for the most rational treatment of the economic phases of the subject.

Synonymy.

The question of generic synonymy and classification also deserves consideration. *Protomyces* and *Schinzia* to which the organism was referred at successive times by Frank can not stand because the fungi first associated with these genera would have precedence over the present one. *Bacillus*, to which Beyerinck referred it, can not be retained, since, as Prazmowski has shown, we do not at present know of an endogenous spore formation, and also for the reason that, even according to Beyerinck, the organism is not a true schizomycete. The latter reason would disqualify *Bacterium*, the location proposed by Prazmowski. Frank's *Rhizobium* is based on a micrococcoid form which at least could only represent a minute form of the organism, leaving out of consideration entirely, as Frank does, the hypha form as a part of his *Rhizobium*.

Laurent is not justified then in emending by expansion a genus based on a micrococcoid form, to include a complex plant, the important characters of which the author of *Rhizobium* says form no genetic connection with his genus. Likewise it can not be justly emended from either Frank's or Laurent's *Rhizobium* to embrace only the bacteroids of the tubercles as Schneider has done.

However, Schrœter's *Phytomyxa* antedates Frank's *Rhizobium*. It is, moreover, a very easy matter to determine the fungus from Schrœter's description of *Phytomyxa*, even though the author may have erred in placing the genus among

the *Myxomyceteæ*. In fact *Phytomyxa* was erected to represent exactly the morphological characters which we find present in the fungus in the tubercles.

Laurent, probably from the analogy of the form of some of the bacteroids and the forked individuals in his artificial cultures to the various stages of longitudinal division, as Metchnikoff[125] terms it, of *Pasteuria ramosa*, places it in the family *Pasteuriaceæ*. There does notseem to be any good evidence that longitudinal division occurs in the organism of the tubercles, as is described for *Pasteuria*, but that these forked forms are derived in an entirely opposite manner, from that which obtains in *Pasteuria*, *i. e.*, by growth instead of longitudinal division, or what seems, more properly speaking, from Metchnikoff's descriptions to be a stellate or radiate division, beginning with numerous invaginations upon the external surface and proceeding toward the center until finally the quadrants and octants present a division approaching the longitudinal.

It still remains to note certain remarkable phenomena observed by the present writer in one of the cell cultures of the vetch tubercle organism. The microscope was focussed upon several individuals representing various lobed forms to observe their development. Sketches were made of the position and form of all the individuals in the field of the microscope in order that their development might be accurately recorded. In the course of twenty-four hours one of the individuals had disappeared from view, and now and then minute motile organisms swept across the field. During that day one of the larger lobed forms disintegrated by the loss in some way, which was not observed because interrupted observations were made, of the dense portions of protoplasm at certain points near the periphery. The most remarkable phenomenon, however, was the fact that one of the individuals, the form of which might be described as representing the union of two clavate bodies by their larger ends, was moving about sluggishly as if drawn by something attached to one end. For ten to fifteen minutes it moved about within the field of the microscope with a slow oscillatory movement combined with the progressive movement, when it disappeared from view. These observations have suggested the possibility of the formation of zoospores within the larger of the individuals in artificial cultures and in the buddings or enlargements of the hyphæ within the tubercle.

[125] Ann. d. l'Institut Pasteur II (1888). 165–170.

Little value is attached by the writer to these observations since it was impossible at that time to repeat them, for in a few days his labors were to be removed from Alabama to New York, and during the move the infusion of *Vicia sativa* with which it was designed to make media to prosecute the study farther became contaminated. During the busy period of organizing work in a new field the organism died before fresh culture media could be made.

Laurent did not observe a motile stage. Beyerinck observed motile forms which agree in size with the smallest forms (0.2μ) obtained by myself. If Frank's micrococci and Prazmowski's swarmers could be regarded as the same forms represented by these small individuals the possibility of there being zoospores would be strengthened. Should the presence of zoospores be confirmed it would indicate a relationship to the lower *Phycomycetes*. It will be remembered that Vuillemin (l. c.) placed the organism in the *Chytridiaceæ*, but the zoospores of his *Cladochytrium tuberculorum* were 7μ in diameter, a size much greater than any except possibly some of the very largest of the organisms obtained by other investigators, and since his studies were made in late autumn on old tubercles there may have been some chance of monads occurring in the tissue of the tubercles.

While in some characters, as noted above, the tubercle organism is very much like *Cladochytrium tenue*, yet in the sum of essential characters it departs too widely from that genus, so that even if it should eventually be clearly shown to be one of the *Chytridiaceæ*, it would still be referable to *Phytomyxa*.

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EXPLANATION OF PLATES XII–XV.

PLATE XII.—Tubercles of Vicia sativa, from photographs.

PLATE XIII.—Fig. 4. Young tubercle, magnified, showing affected root hair. —Fig. 5. Same root hair more magnified, showing form of infecting thread.—Fig. 6. Another view of fig. 5.—Fig. 7. Section of outer portion of tubercle with root hair, showing entering infecting thread.—Fig. 8. Section of young tubercle with infecting thread *in situ* showing enlargement and buds; *en*, endodermis; *pe*, pericambium; *ph*, phloem; *x*, xylem.

PLATE XIV.—Fig. 9. Infecting thread drawn to larger scale.—Fig. 10. Portion of bacteroid tissue containing branching threads of *Phytomyxa; n*, nucleus of tubercle cells.—Figs. 11 and 12. Organisms in pure culture of *Phytomyxa* from vetch tubercle.—Fig. 13. Bacteroids of *Vicia sativa* tubercle.—Figs. 14 and 15. Bacteroids of *Medicago denticulata* tubercle. The scale in 13, 14 and 15 is $\frac{1}{2}$mm, and the objects are magnified thirty times more than the scale.

PLATE XV.—Inoculation. Water culture of *Vicia sativa*. Nos. 1 and 4 inoculated with organisms from pure culture of *Phytomyxa* from *Vicia sativa* tubercles. From photograph.